

Ethanol-Induced Inflammation in the Developing Hippocampus:

Mast Cell Degranulation and Microglia Activation

Research Thesis

Presented in partial fulfillment of the requirements for graduation
with Research Distinction in Neuroscience in the Undergraduate
Colleges of The Ohio State University

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April 2017

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Abstract

Fetal alcohol spectrum disorders (FASD) refer to a range of physical and mental deficits resulting from maternal alcohol consumption during pregnancy. Ethanol exposure leads to a neuroinflammatory response mediated through the activation of microglia, the brain's resident immune cell. While microglia have been the focus of numerous neuroinflammation research studies, much less is known regarding ethanol's effects on another resident brain cell, mast cells (MCs). MCs are found throughout the brain, including the hippocampus, and exist in two phenotypical states: a stable granulated state and an active degranulated state. MCs, not microglia, are considered to be "first responders" to a toxic event, including, possibly, ethanol exposure. Activation of MCs leads to rapid degranulation and the release of a number of preformed or newly synthesized pro-inflammatory cytokines, including TNF α and IL-6. Activated brain MCs are proposed to drive microglial activation and the exacerbation of neuroinflammation. In this study, rat pups were intragastrically intubated with ethanol (5g/kg/day) or sham intubated over postnatal days (PD) 4-6. Thirty minutes prior to each day's intubation procedure pups were cryoanesthetized and bilaterally injected into the lateral ventricles with cromolyn, a MC degranulation inhibitor. All subjects were sacrificed on PD 6, two hours after ethanol administration, and the brains submitted to immunohistochemical processing for MCs and microglia. Results indicate that postnatal ethanol exposure induced significant MC degranulation and microglia activation in both the dorsal and ventral hippocampus, relative to sham-intubated and unhandled controls. Cromolyn injections attenuated MC degranulation and the morphological activation of microglia. The interaction between these two neuroimmune cells could lead to novel therapeutic treatments for those diagnosed with FASD.

Introduction

Fetal Alcohol Spectrum Disorders (FASD) describe a range of cognitive and physical abnormalities resulting from maternal alcohol consumption during pregnancy. In the US approximately 5% of American school-aged children are affected by FASD (May et al., 2009), which leads to an economic cost of approximately \$4 billion annually (Lupton et al., 2004). Children who suffer from FASD are more likely to show high comorbidity for mental health deficits, including attention deficit hyperactive disorder (ADHD), as well as social impairments, such as school delinquency (Streissguth et. al., 2004). Furthermore, children afflicted with FASD possess smaller, asymmetrical hippocampi (Riikonen et. al. 1999) and demonstrate deficits in hippocampal-dependent cognition (Dudek et al., 2014).

The hippocampus is responsible for declarative learning and memory. It is typically separated into two portions with different functional capabilities the dorsal hippocampus, which is involved in cognitive learning and memory and the ventral hippocampus, which is associated with emotional behavior (Fanselow and Dong 2010). The hippocampus is composed of three sub-regions: CA1, CA3, and the dentate gyrus (DG). Past work in animal models of FASD have shown the hippocampus is susceptible to the deleterious effects of pre- and/or postnatal ethanol exposure including disrupted neurogenesis, decreased dendritic formation, impeded synaptogenesis, and neuron death (Klintsova et al., 2007; Tran and Kelly, 2003; Pascual et al., 2007). Combined, these deficits lead to diminished hippocampus functionality. Modeling FASD, our lab administers ethanol to rats during early postnatal life, a human third trimester equivalent period, which leads to long-lasting impairments in hippocampal-dependent learning and memory (Goodfellow et al., 2014, Dupont et al., 2014).

Early-life ethanol exposure also leads to a neuroinflammatory response mediated through the activation of microglia (Alfonso-Loeches et al., 2015), one of the brain's resident immune cells. Microglia respond to an immune insult through the release of either pro-inflammatory cytokines (M1 phenotype), which propagate neuroinflammation, or the release of anti-inflammatory cytokines (M2 phenotype) that work to alleviate neuroinflammation and promote repair (as reviewed in Kempuraj et al., 2016). Recent work in rodents demonstrates that ethanol exposure provokes a neuroinflammatory response through the activation of Toll-like receptor 4 (TLR-4) and corresponds with the activation of microglia and the release of pro-inflammatory cytokines (Pascual et al., 2007; Fernandez-Lizarbe et al. 2008; Crews et al. 2014). Microglia can be found in four morphological states. Quiescent microglia exhibit a thin ramified state in which their processes survey the surrounding extracellular space. Once activated, they begin a transition, first into a thick ramified state and then into a bushy state. A microglia is fully activated when it reaches the amoeboid state (Schwarz et al., 2012). In FASD model rats, ethanol exposure has been shown to increase the proportion of microglia with an amoeboid phenotype, which leads to the increased release of both pro- and anti-inflammatory cytokines (Drew et al., 2015; Boschen et al. 2016). The release of pro-inflammatory cytokines, including interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF- α), and subsequent increases in neuroinflammation can induce neurodegeneration and neuronal death and ultimately impede cognition (as reviewed in Minogue et al., 2017, and Kempuraj et al., 2016).

While microglia have been the focus of numerous neuroinflammation research studies (e.g., Ahmad et al., 2015), little to nothing is known regarding ethanol's effects on

mast cells (MCs), another resident immune cell of the brain. MCs are part of the innate immune system, which are formed from hematopoietic stem cells in bone marrow (Gilfillan et al., 2011). MCs travel to the brain through the vasculature and almost 97% of MCs reside within the brain (Jin et al., 2009; Khalil et al., 2007). Central MCs are primarily found in two states: a stable “granulated” state and an activated “degranulated” state. Due to the MC’s ability to rapidly degranulate, it is suggested that they act as “first responders” to illness or infection (Lindsberg et al., 2010). MCs are activated by pro-inflammatory cytokines, bacterial and viral products, toxins and other neuroimmune related signals (as reviewed in Silver et al., 2013) including, this proposal aims to demonstrate, ethanol. We propose that ethanol activates MCs through its interactions with TLR-4 (as reviewed in Skaper et al., 2012). This activation causes MCs to degranulate, releasing their granule contents, including histamine, serotonin, and the pro-inflammatory cytokines TNF- α and IL-6 (Skaper et al., 2012), through the exocytosis of cytoplasmic granules (i.e. small particles) into the extracellular space (Gordon et al., 1991). These granule contents can be either preformed and rapidly released, or released over a span of hours through *de novo* synthesis allowing for sustained MC activation (Gordon et al., 1991; Gu et. al. 2015). Degranulation can induce an acute inflammatory response (Cocchiara et al., 1997), as well as “recruit” other immune cells (Dong et al., 2016). MCs may do this through the rapid release of preformed TNF- α followed by *de novo* synthesis and release (Gu et. al. 2015), which can lead to upregulated numbers and activation of microglia and other MCs (Dong et. al. 2014, Gu et. al. 2015).

Several mechanisms for interactions between MCs and microglia have been shown *in vitro* (Li et al., 2017). One hypothesized mechanism is that MC tryptase activates

protease-activated receptor 2 (PAR-2) on microglia, leading to an increase of pro-inflammatory cytokines and reactive oxygen species (ROS) thereby promoting neuroinflammation (Zhang et. al. 2012). However, MC and microglia interactions *in vivo* are relatively unexplored (Dong et. al. 2016).

To study the effects of MC degranulation on microglia activation we use a MC degranulation inhibitor, disodium cromoglycate (cromolyn). Cromolyn stabilizes the membrane of MCs, possibly through decreasing the influx of calcium (Alton et al., 1996), which prevents degranulation (as reviewed in Brogden et al., 1974). Cromolyn injections have been shown to attenuate MC degranulation in the hippocampus and other brain regions (Dong et al., 2016, Valle-Dorado et al., 2015).

This study investigates the effects of postnatal days (PD) 4-6 ethanol exposure on the morphological state of MCs and microglia in the developing hippocampus. Ethanol is thought to directly activate TLR-4, found on the surface of both MCs and microglia, or act upon it indirectly via the release of high-mobility box group 1 (HMGB1), an endogenous agonist of TLR-4 (Crews et al, 2015; Crews et al., 2014, Skaper et al., 2012). PD 4-6 ethanol is proposed to increase MC degranulation, which amplifies or drives the morphological activation of microglia (Figure 1). The administration of cromolyn is hypothesized to decrease the proportion of degranulated MCs and thereby lower the percentage of morphologically activated microglia. This research is particularly significant because there has been no FASD research published to date looking at the effects of perinatal ethanol exposure on MCs, and the subsequent recruitment of microglia.

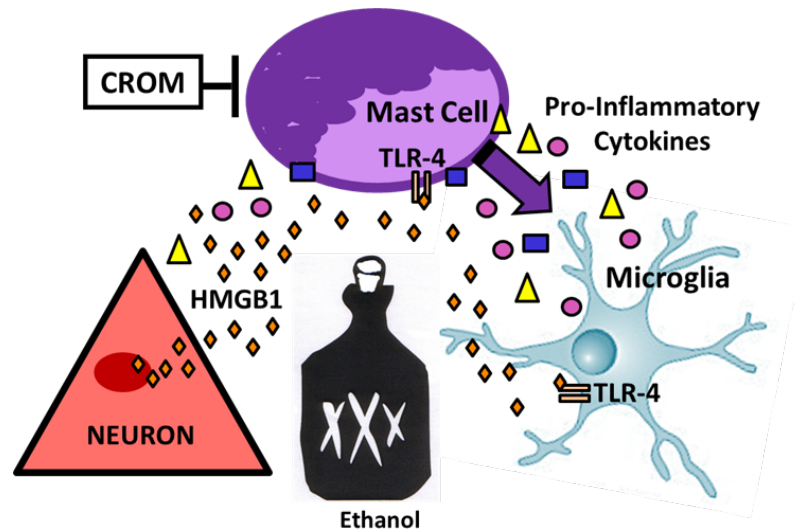


Figure 1

This study investigates the deleterious effects of postnatal ethanol's interaction with MCs and microglia via TLR-4 activation. Ethanol is proposed to directly activate TLR-4 or indirectly activate it via the release of HMGB1, which acts as a TLR-4 agonist. MC degranulation amplifies microglia activation, which leads to further cytokine release. Increased MC and microglia activation can produce persistent feed-forward loops that underlie chronic neuroinflammation.

Methods

Subjects

Male and female Long-Evans rats, purchased from Envigo Laboratories (previously Harlan, Indianapolis, IN), were housed in the Psychology department vivarium at The Ohio State University, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) -accredited facility. The rats were placed on a 12-hour light/dark cycle (lights on at 0600 hr) with ad-lib access to food and water. Male and female breeder rats were paired for 1 week. After three weeks, the dams were checked daily, in the morning and evening, for parturition. Litters were culled to a maximum of 12 subjects on PD 3, keeping male and female numbers even when possible. This experiment includes only male subjects; female subjects from the same litter were selected for alternative research experiments. All procedures were in strict compliance with the Ohio State University Institutional Animal Care and Use Committee (IACUC) animal care guidelines, and all

necessary measures were taken to minimize pain and discomfort. On PD 3, subjects were placed in a plastic container on top of a heating pad and weighed. Subjects were then injected with non-toxic black ink into one or more paws, using a sterile syringe, for identification purposes. This thesis used a within subjects design for quantifying MC and microglia numbers and activation state. In total 20 rats were used for this experiment.

Intubations

On PD 4, 5, and 6, subjects were administered binge-like doses of ethanol (5E) via intragastric intubations, or sham intubations (SI) in which no ethanol was administered. Starting at approximately 0900 hr subjects were removed from their home cage and weighed. Then, PE10 plastic tubing, lubricated with corn oil, was lowered down the esophagus and into the stomach of the subject. A mixture of nutritive milk (West et. al. 1986) and ethanol (11.33% ethanol), at a dose of 5g/kg/day based on the subject's body weight (0.02778 mL/g), was then administered through the tubing via a 1 mL syringe. Following a 2-hour waiting period 5E subjects were intubated again, this time using a nutritive milk only solution in order to support weight gain, which can be impacted due to inebriation and its negative impact on regular nursing. On PD 4 alone, 5E rats were submitted to a second milk-alone intubation after another 2-hour waiting period. SI controls were submitted to intubations at equal times and numbers, though no solutions were ever administered. Unhandled control (UC) rats were removed from the dam with the rest of the litter and weighed daily, but not submitted to intubations. The entire intubation process took approximately 10-15 minutes per litter, after which the subjects were returned to the dam and the home cage returned to the vivarium subsequently.

Intracerebral Ventricular Injections

Approximately 40 minutes prior to intubations on PD 4-6, subjects were submitted to bilateral intracerebral ventricular (ICV) injections. Subjects were anesthetized via cryoanesthesia, a procedure that leads to no later-life behavioral deficits in mice pups (Janus et al., 2014). The subjects were enveloped in aluminum foil, to protect them from direct contact with ice, placed in a cooler filled with ice and set in a 4° C refrigerator for approximately 15 minutes. Anesthesia was verified via toe-pinches and for surgery, the subject's head was alternatively swabbed (3x) with Betadine and 70% ethanol. The subject, atop the icepack, was then moved into a stereotaxic apparatus. A 10 μ L Hamilton needle with a beveled end, sterilized via a dry sterilizer (Germinator [™] 500) was affixed to the stereotax and filled with either cromolyn (dosage: 100 μ g/ μ L) (Dong et al., 2016) (Sigma-Aldrich, St. Louis, MO) or 0.9% sterile saline as a control. The tip of the needle was then aligned over Bregma and moved over the lateral ventricles (-1mm A/P, +/- 1mm M/L). The scalp and skull during this point in development are thin and no incision or drilling was required. Thus the needle was lowered through the subject's scalp and skull (-3 mm D/L), into the lateral ventricles, and raised 0.5 mm in order to create a space in which the injected solution could diffuse. A total of 1.5 μ L of solution was dispensed and allowed to diffuse for 3 minutes before the needle was removed and the other hemisphere submitted to the same procedure. The injection procedure lasted approximately 10 minutes, after which the subject was set on a towel on top of a heating pad, and stimulated with human touch, without gloves, to allow for heat transfer. Full recovery took approximately 15-20 minutes and after normal behavior and the ability to swallow were confirmed, the subject

was returned to its litter and submitted to intubations. Behavior was assessed by monitoring the animal's alertness via its response to touch while its ability to swallow was checked by inserting intubation tubing into the subject's mouth and determining a swallowing reflex (Janus et. al. 2014).

Tissue Collection

On PD6, the subjects were placed into an induction chamber and deeply anesthetized with isoflourane, for approximately 1-2 minutes. The subjects were removed from the chamber, a pneumothorax was completed, and subjects were submitted to a transcardial perfusion in which 25 mL of ice cold 0.9% saline was administered followed by 4% paraformaldehyde (PFA). After the subject was perfused, it was decapitated, its brain removed, and stored in 4% PFA in a 4°C refrigerator to fix overnight. The following day the brain was placed in 30% sucrose, 4% PFA inside of a 4°C refrigerator until the brain sank to the bottom of the solution. Following this fixing process the brain was sectioned (coronal; 50 µm) using a VT1000s vibratome (Leica Biosystems, Buffalo Grove, IL) across the full extent of the hippocampus was. The sections were plated onto gel coated glass slides (for MC staining) and every eighth section was placed into a well, filled with cryoprotectant, for microglia immunohistochemical (IHC) processing.

Toluidine Blue Staining

MC slides were dyed with toluidine blue, a positive basic dye that colors MCs blue because of their acidic nature, in order to identify and quantify MCs in and surrounding the hippocampus. This process occurs by first submerging the slides in 60% EtOH for 2

minutes followed by a 4:1 mixture of acidified (pH 2.0) 60% EtOH and 5X toluidine stock solution for 20 minutes. This stock solution was made by mixing 2.5 g of Toluidine Blue powder with 100 mL of 60% EtOH. The slides were then dehydrated by submerging them in 50% EtOH for 15 seconds, 70% EtOH for 45 seconds, 95% EtOH for 2 minutes, and 100% EtOH for 2 minutes. Lastly, the slides were submerged in Histo-Clear® (National Diagnostics, Atlanta, GA) for 10 minutes, cover slipped with Cytoseal® (Thermo Scientific, Waltham, MA) and left to dry overnight in the fume hood.

Immunohistochemical Processing

Every eighth section of each subject's hippocampus was submitted to IHC processing in order to determine the morphological state of microglia. IHC is a three-day procedure; on the first day free-floating sections were rinsed 3 x 5 minutes in phosphate buffered saline (PBS). Sections were blocked for 1 h in 10% bovine serum albumin in PBS + 0.4% Triton-X (PBST) and then incubated in 50% methanol + 0.3% H₂O₂ for 1 hour, at 4°C and rinsed in PBS 3 x 5 minutes. Then, sections were placed in the primary antibody, anti-Iba-1 (1:1000, Wako, Richmond, VA), diluted in blocking buffer overnight at 4°C. The following morning sections were rinsed 3 x 20 minutes in PBS, which was followed by a 1-hour incubation period in the secondary antibody (Vector biotinylated anti-rabbit 1:500) in blocking solution. Following a 3 x 5 minute rinse cycle of PBST, sections were placed in an ABC complex (Vector Laboratories, Burlingame, CA) for one hour, then rinsed 3 x 5 minutes in PBST followed by a 3 x 5 minute rinse cycle of 0.175 Sodium Acetate Buffer. After this process was complete the sections were submitted to a rinse in a chromagen DAB substrate working solution for approximately 2 minutes followed by a 3 x 5 minute rinses in PBS.

Sections were mounted in diluted sodium acetate in water onto gel-coated glass slides, and left in the fume hood to dry overnight. The next day the slides were soaked in ddH₂O for 5 minutes, dehydrated, and submerged in Histo-Clear® for 10 minutes until coverslipped with Cytoseal®.

Microscopy

Following staining protocols, all sections were viewed using a Zeiss Axio Imager M2 for bright field microscopy. The program Stereo Investigator (v.11) (MBF biosciences, Williston, VT) was used to image sections. MC and microglia sections were analyzed using two different methodologies, detailed below. During all analyses the researcher was kept blind to the subject's treatment group.

Mast Cell Counts

Approximately 35 sections per subject were allocated for MC counts. MCs were counted in the dorsal and ventral hippocampus, as well as the brain region surrounding the lateral ventricles, including the velum interpositum (Stanley et al., 1990) (Figure 2A), where MCs are commonly located. MCs were counted at 10x magnification in both hemispheres of the section. The number and location of each MC was also recorded and classified as either granulated or degranulated (Figure 2A, B). MCs were considered degranulated when they met one or more of the following criteria: loss of purple staining, fuzzy appearance, or exceedingly distorted shape (Zhang et. al. 2016).

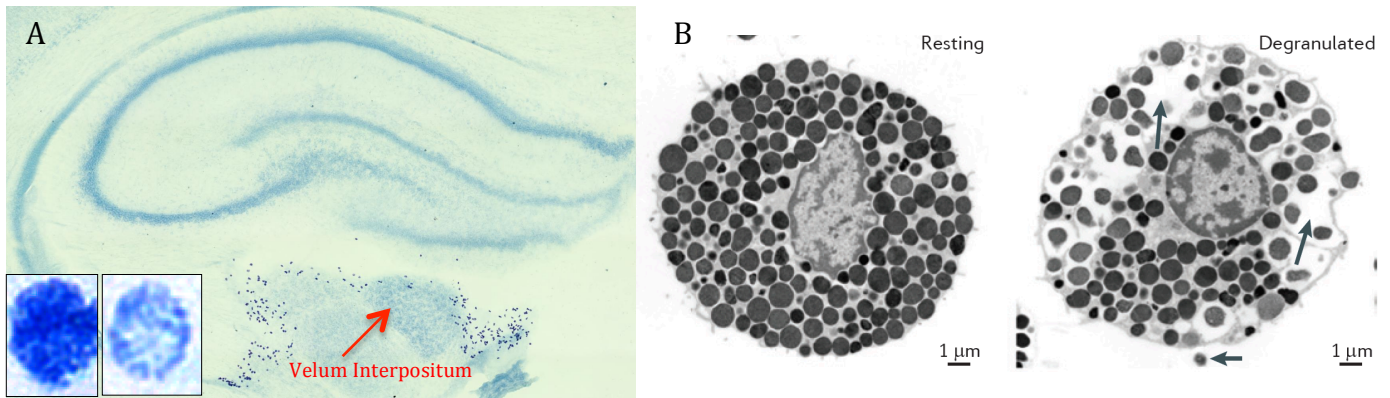


Figure 2

A) Photomicrograph (2.5x) depicting the location of MCs in the dorsal hippocampus and velum interpositum. Inset shows photomicrographs (40x) depicting granulated (left) vs degranulated (right) MCs. B) Image depicting the differences between granulated (left) vs degranulated (right) MCs. Long arrows indicate where granule contents have been exocytosed, while the short arrow indicates a granule content that has been released from the MC (image from Wernersson et al., 2014).

Microglia Morphological Counts

Every eighth section of the subject's brain was allocated for microglia morphology with an average of 5 sections per brain analyzed. Microglia morphology was determined in both the dorsal and ventral hippocampus within one hemisphere per section, which was randomly selected for analysis. Regions of interest (ROIs) were drawn around areas CA1, CA3 and DG, with a 2.5x objective, using the Optical Fractionator Workflow program (MBF biosciences, Williston, VT). Cell counts were performed at 20x to distinguish the different morphological phenotypes. Before counting began the sections mounted thickness was measured, the counting frame size was determined (200 x 200μm), and the grid size was adjusted so that ~10x counting frames were generated per ROI. Microglia were categorized by morphological phenotype into four groups (Schwarz et al., 2012): thin ramified, thick ramified, bushy, and amoeboid (Figure 3B).

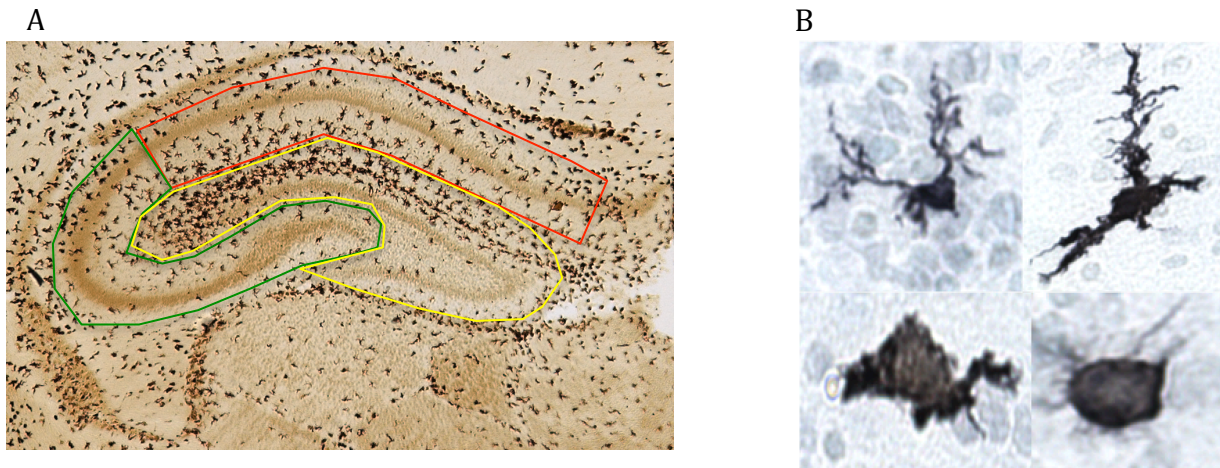


Figure 3

A) Photomicrograph depicting microglia in the sub-regions of the dorsal hippocampus: CA1 (red) CA3 (green) DG (yellow). B) Photomicrograph (60X) depicting four microglia phenotypes: thin ramified (top-left), thick ramified (top-right), bushy (bottom-left), and amoeboid (bottom right).

Statistical Analysis

Data were analyzed via single-factor analyses of variance (ANOVA) tests and, when appropriate, Fisher's LSD post-hoc tests (significance implies $p < 0.05$). MC counts were performed in 19 male rats: UC ($n = 4$), SI-Veh ($n = 5$), 5E-Veh ($n = 4$), 5E-Crom ($n = 6$). Microglia analyses were performed in 18 male rats: UC ($n = 3$), SI-Veh ($n = 5$), 5E-Veh ($n = 5$), 5E-Crom ($n = 5$).

Results

Mast Cells Counts

Total MC numbers in the hippocampus were analyzed using single factor (treatment) ANOVAs. No significant differences were seen between treatment groups (Figure 4A). The hippocampus was then divided into two sections, dorsal and ventral, and single factor (treatment) ANOVAs run, which again found no significant differences between treatment groups (Figure 4B). Next, MCs inside the hippocampus and outside (i.e.,

inside of the velum hippocampus, surrounding the lateral ventricles) were compared using single factor (treatment) ANOVAs, which found no significant differences between treatment groups in either area (Figure 4C). Following these findings, data was analyzed to compare MCs located either inside or outside of the dorsal (Figure 4D), and ventral (Figure 4E) hippocampus. Single factor (treatment) ANOVAs again showed no significant differences in total MC numbers in any treatment group. Finally, MCs were distinguished as either granulated or degranulated across the whole hippocampus and total numbers were analyzed using single factor (treatment) ANOVAs, which revealed significant treatment group differences in degranulated MC numbers $F(3, 15) = 4.77, p < 0.05$ (Figure 4F). Fischer's LSD post-hoc analysis revealed an elevated number of degranulated MCs in 5E-Veh rats when compared to all other treatment groups (UC, SI-Veh, and 5E-Crom).

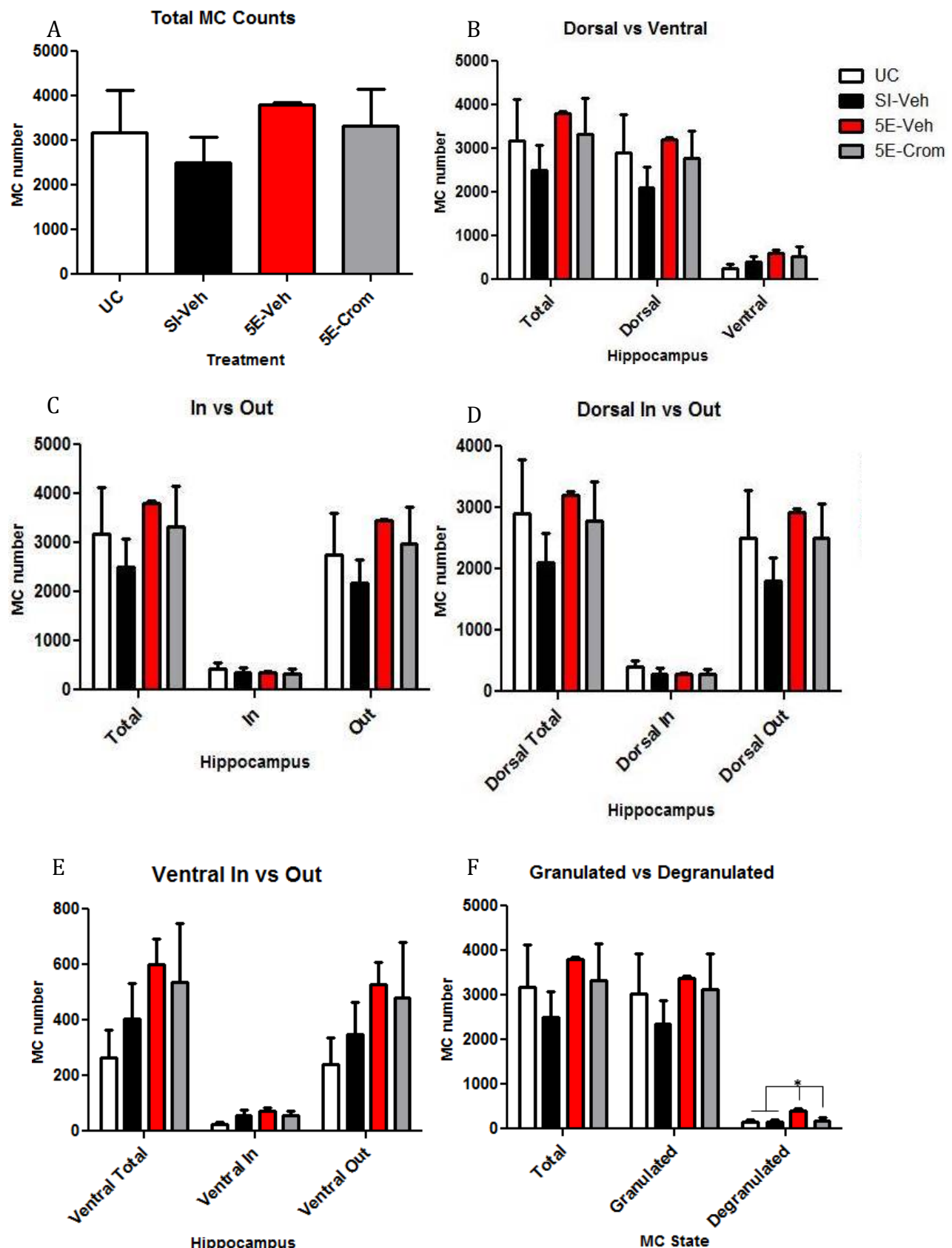


Figure 4

A) Ethanol exposure and cromolyn administration exerted no significant effects on MC number between groups. B) MCs are located primarily in the dorsal hippocampus when compared to ventral. C) MCs are located primarily outside of the hippocampus in comparison to inside. D) MCs are located primarily outside of the dorsal hippocampus in comparison to inside. E) MCs are located primarily outside the ventral hippocampus in comparison to inside. Ethanol exposure and cromolyn administration exerted no significant effects on total number of MCs, relative to controls, inside or outside of the dorsal hippocampus. F) Across the whole hippocampus there was a significant increase in the number of degranulated MCs in 5E-Veh rats, which was significantly attenuated by cromolyn administration in 5E-Crom rats ($p < .05$).

Mast Cell Percent Degranulation

After calculating the percentage of MC degranulation for the developing hippocampus, single factor (treatment) ANOVAs revealed significant differences in MC degranulation percentages between treatment groups $F(3, 15) = 10.16, p < 0.001$. Post-hoc analysis indicated that the percentage of MC degranulation in 5E-Veh rats was significantly increased when compared to all other treatment groups (Figure 5A). The degranulation percentage was then calculated for the dorsal and ventral hippocampus, and single factor (treatment) ANOVAs revealed statistically significant effects of ethanol exposure: dorsal $F(3, 15) = 7.34, p < 0.01$, ventral $F(3, 15) = 49.95, p < 0.001$. Post-hoc analyses revealed significant increases in 5E-Veh MC degranulation percentages relative to all other groups in both the dorsal and ventral hippocampus (Figure 5A). Finally, single factor (treatment) ANOVAs revealed significant differences in MC degranulation percentage, both inside and outside of the dorsal (Figure 5B), ventral (Figure 5C), and total hippocampus (Figure 5D): dorsal hippocampus, in: $F(3, 15) = 16.77, p < 0.001$, out: $F(3, 15) = 6.55, p < 0.01$), ventral hippocampus, in: $F(3, 15) = 14.03, p < 0.001$, out: $F(3, 15) = 36.40, p < 0.001$), and total hippocampus, in: $F(3, 15) = 22.99, p < 0.001$, out: $F(3, 15) = 28.47, p < 0.001$). Post-hoc analyses indicate MC degranulation was significantly elevated in 5E-Veh subjects, relative to all other treatment groups, across all analyzed regions.

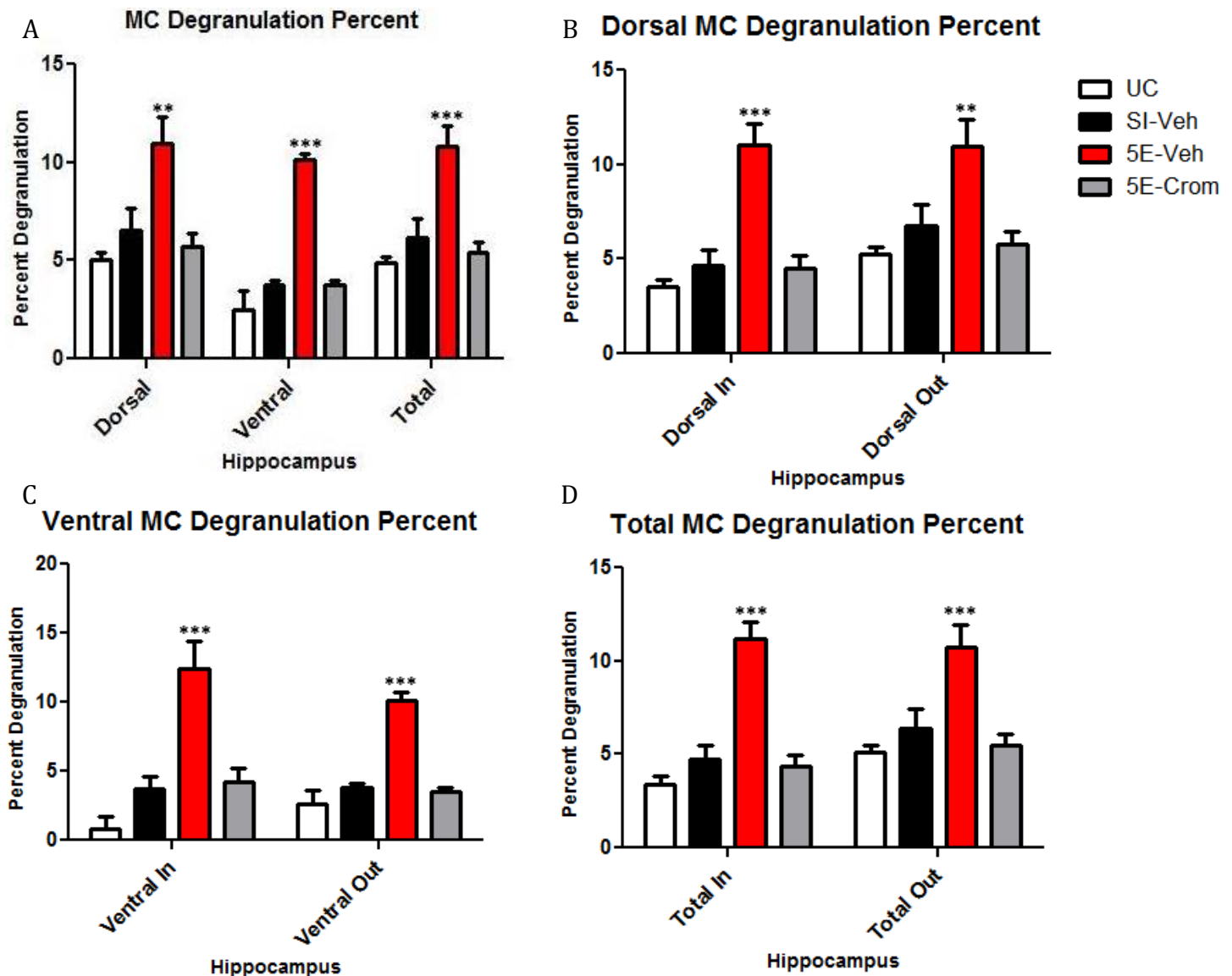


Figure 5

A) The percentage of degranulated MCs is significantly increased in the dorsal ($p < .01$), ventral ($p < .001$), and total ($p < .001$) hippocampus of 5E-Veh subjects relative to controls. Cromolyn administration in 5E-Crom rats significantly attenuated this effect. MC degranulation is significantly increased inside and outside of the B) dorsal (in: $p < .001$, out: $p < .01$) C) ventral ($p < .001$) and D) total hippocampus ($p < .001$) in 5E-Veh rats. Cromolyn administration in 5E-Crom rats significantly attenuated this effect.

Microglia

Total microglia numbers from select ROIs within the trisynaptic hippocampal circuit were analyzed using single factor (treatment) ANOVAs, which revealed no significant

differences in microglia number across treatment groups (Figure 6A). Microglia were then analyzed by their morphological phenotype as either thin, thick, bushy, or ameboid. First, the percentage of each phenotype was analyzed across the entire hippocampus using single factor (treatment) ANOVAs which indicated significant differences in the percentage of thin ramified $F(3, 14) = 9.83, p < 0.001$, thick ramified $F(3, 14) = 4.76, p < 0.05$, and ameboid microglia $F(3, 14) = 4.67, p < 0.05$. Post-hoc testing revealed a significant increase in thin ramified microglia in UC subjects over all other treatment groups, and a corresponding decrease in the percentage of thick ramified microglia in UC subjects in comparison to all treatment groups. Importantly, the percentage of ameboid microglia was significantly increased in 5E-Veh subjects over all other treatment groups (Figure 6B), suggesting postnatal ethanol induced an activated phenotype.

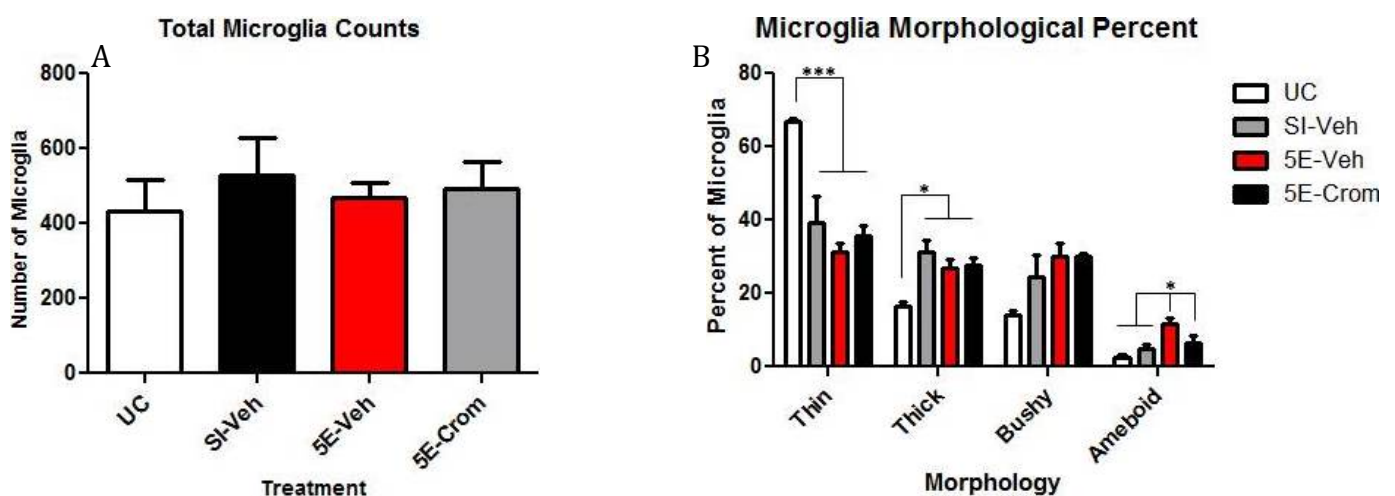


Figure 6

A) Ethanol exposure and cromolyn administration exerted no significant effects on microglia number, relative to controls, in the neonate rat hippocampus. B) Significant decreases in the percentage of thin ramified microglia ($p < .001$), paired with significant increases in the percentage of thick ramified microglia ($p < .05$) seen in UC rats relative to controls. The 5E-Veh rats exhibited a significant increase ($p < .05$) in the percentage of ameboid microglia relative to controls, which was significantly attenuated by cromolyn administration in 5E-Crom rats.

The hippocampus was divided into its dorsal and ventral divisions and the percentage of each microglia phenotype was analyzed in three areas: CA1, CA3, and DG.

The percentage of thin ramified microglia were analyzed using single factor (treatment) ANOVAs, which found significant treatment group differences in all three sub-regions of the dorsal hippocampus, CA1: $F(3, 14) = 3.98$, $p < 0.05$, CA3: $F(3, 14) = 4.76$, $p < 0.05$, and DG: $F(3, 14) = 7.87$, $p < 0.01$. Post-hoc analysis revealed a significant increase in the percentage of thin ramified microglia in UC subjects when compared to all other treatment groups (Figure 7A). Single factor (treatment) ANOVAs were then used to analyze the percentage of thick microglia in all three sub-regions of the dorsal hippocampus, which found significant effects in area CA3, $F(3,14) = 5.94$, $p < 0.01$. Post-hoc analysis determined a significantly lower percentage of thick ramified microglia in UC subjects compared to all other treatment groups (Figure 7B). The percentage of bushy microglia was analyzed using single factor (treatment) ANOVAs, which found no significant differences (Figure 7C). Finally, the percentage of ameboid microglia in all three sub-regions of the dorsal hippocampus was analyzed using single factor (treatment) ANOVAs, which found a statistically significant treatment group differences in the DG, $F(3,14) = 5.61$, $p < 0.01$. Post-hoc testing indicated the percentage of ameboid microglia in 5E-Veh subjects was significantly increased compared to all other treatment groups (Figure 7D).

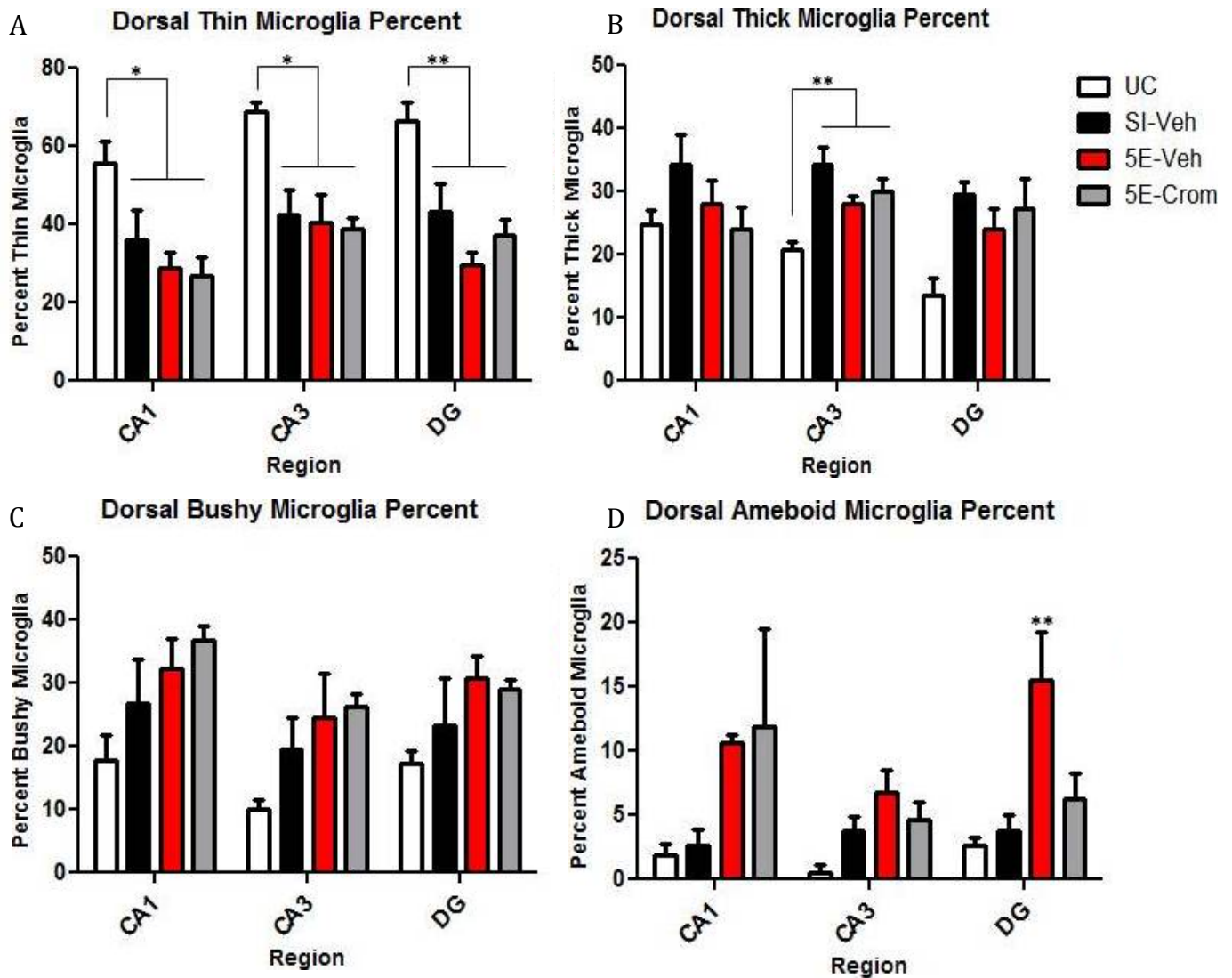


Figure 7

A) Significant decreases in the percentage of thin ramified microglia across all sub-regions of the dorsal hippocampus, CA1 ($p < .05$), CA3 ($p < .05$), and DG ($p < .01$) in all subjects relative to UC rats. B) There is a significant increase in the percentage of thick ramified microglia in area CA3 ($p < .01$) in all subjects relative to UC rats. C) Ethanol exposure and cromolyn administration exerted no significant effects on the percentage of bushy microglia in any sub-region of the dorsal hippocampus relative to controls. D) Ethanol exposure induced a significant increase in the percentage of ameboid microglia in the DG ($p < .01$) sub-region of the dorsal hippocampus of 5E-Veh rats in comparison to controls. Cromolyn injections significantly attenuated this effect in 5E-Crom rats.

Similar analyses were performed to determine the percentages of each microglia phenotype in the sub-regions of the ventral hippocampus. All analyses were performed using single factor (treatment) ANOVAs to determine significance. Analysis of the percentage of thin ramified microglia in the ventral hippocampus revealed significant treatment group differences in all three sub-regions, CA1: $F(3, 13) = 4.82$, $p < 0.05$, CA3: $F(3, 13) = 6.84$, $p < 0.01$, and DG: $F(3, 13) = 11.13$, $p < 0.001$. Post-hoc analysis indicated an

increase in the percentage of thin ramified microglia in UC subjects compared to all other treatment groups (Figure 8A). The analyses of both thick ramified (Figure 8B) and bushy microglia (Figure 8C) in revealed no significant effects in any sub-region of the ventral hippocampus. Finally, analysis of the percentage of ameboid microglia revealed significant differences in areas CA3 $F(3,13) = 3.42$, $p < 0.05$, and the DG $F(3,13) = 9.57$, $p < 0.001$. Post-hoc analysis revealed significant increases in the percentage of ameboid microglia in 5E-Veh subjects compared to all other treatment groups in both sub-regions (Figure 8D).

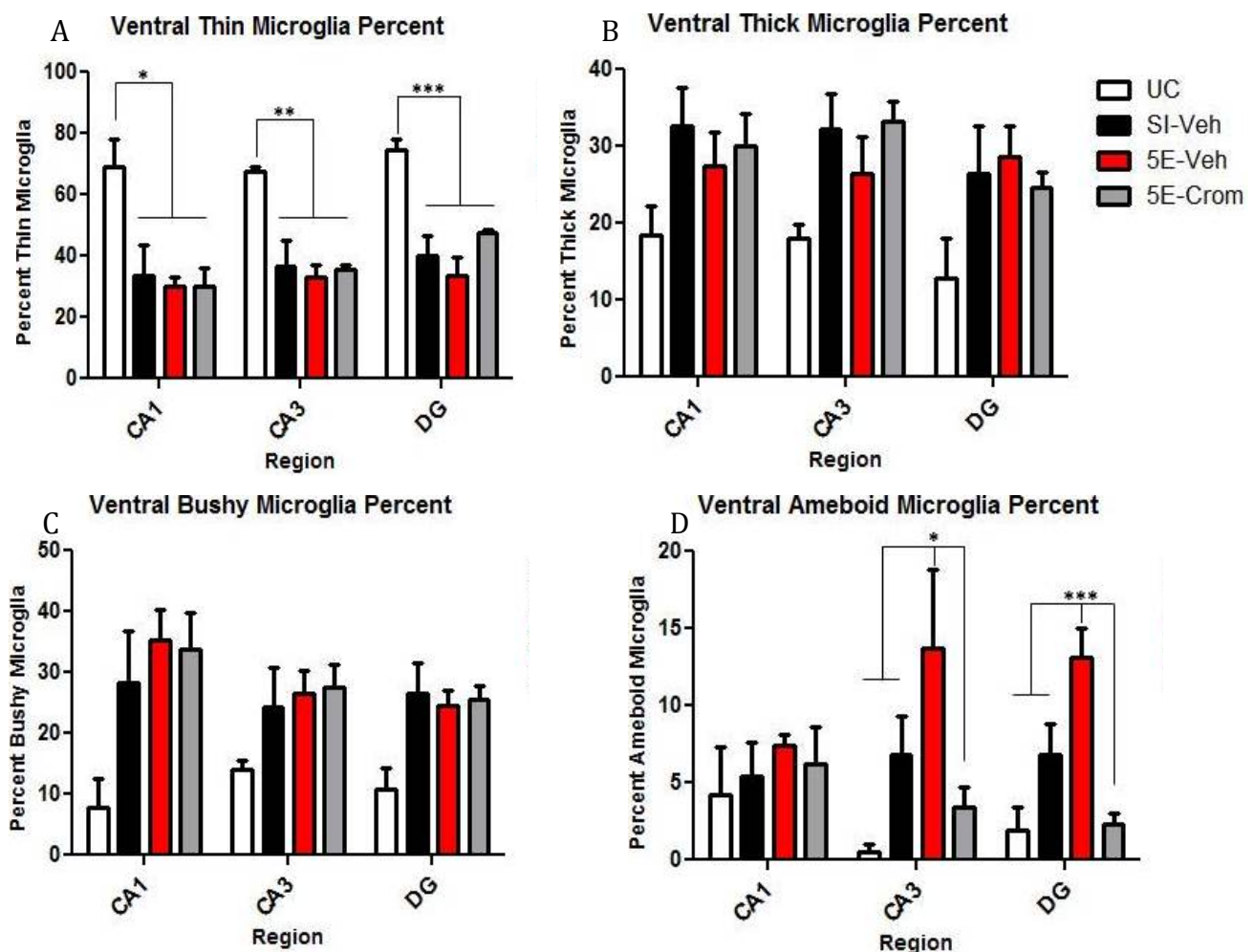


Figure 8

A) Significant decreases in the percentage of thin ramified microglia across all sub-regions of the ventral hippocampus, CA1 ($p < .05$), CA3 ($p < .01$), and DG ($p < .001$) in all subjects relative to UC rats. B) Ethanol exposure and cromolyn administration exerted no significant effects on the percentage of thick ramified microglia in any sub-region of the ventral hippocampus relative to controls. C) Ethanol exposure and cromolyn administration exerted no significant effects on the percentage of bushy microglia in any sub-region of the ventral hippocampus relative to controls. D) Ethanol exposure induced a significant increase in the percentage of ameboid microglia in area CA3 ($p < .05$) and DG ($p < .01$) of the ventral hippocampus in 5E-Veh rats compared to controls. Cromolyn injections significantly attenuated this effect in 5E-Crom rats.

Discussion

This thesis investigated the neuroinflammatory effects of PD 4-6 ethanol exposure, on the developing hippocampus, and its impact on the degranulation of MCs and morphological activation of microglia, two types of neuroimmune cells. We hypothesized that postnatal ethanol exposure would increase MC degranulation and microglia activation in the developing hippocampus, effects that would be attenuated by cromolyn administration via ICV injections. Results indicate that 5E-Veh rats had significant increases in the percentage of hippocampal MC degranulation and percentage of activated microglia, both of which were significantly reduced following ICV cromolyn administration in 5E-Crom rats. To our knowledge, the interactions between third trimester equivalent ethanol exposure, MC degranulation, and microglia activation, have not been previously investigated in the FASD literature.

Nobel Laureate Paul Ehrlich first described MCs in 1878 and depicted their size (6-12 μ m) histochemical properties, and phenotypes (Beaven 2009). In humans, MCs originate from CD34⁺/CD117⁺ pluripotent stem cells within the bone marrow (Gilfillan et al., 2011) while in rodents, the c-kit gene (KIT) is necessary for MCs to fully mature (Tsai et al., 2011). Following their formation, MCs migrate from the bone marrow, into the bloodstream, and into tissue either in the CNS or periphery, where they mature and differentiate into three main subtypes based on location: mucosal, serosal, or brain (Metcalf et al., 1997). This thesis focused on the brain resident MCs, whose numbers peak in the developing hippocampus on ~PD 5 (Nautiyal et al., 2012). Throughout the brain, MCs are found most prominently in the thalamus and hippocampus, and numbers vary

considerably depending on different experimental methodologies, although some studies suggest that adult rats may have up to 45,000 MCs (Lambracht-Hall et al., 1990).

The most commonly studied mechanism of MC activation is through the binding of immunoglobulin type E (IgE) to F_{ce}RI, which is associated with allergic reactions and increases in inflammation. MCs also degranulate when they come into contact with ligands, which bind to Toll-like Receptors (TLRs) and interleukin (IL) receptors (Silver and Curley 2013). Bacterial, viral, and parasitic products, as well as toxins lead to MC degranulation signifying that MCs play a critical role in the innate immune system (as reviewed in Glifillian et al., 2012).

MCs act as “first responders” in the brain (Lindsberg et. al., 2010) and, upon activation, rapidly degranulate and release their granule contents, which can travel up to approximately 50µm (Nautiyal et al., 2009). These granule contents may contain histamine, serotonin, various growth factors, and cytokines, including TNF-α and IL-6. These products, which are both pro- and anti-inflammatory, can lead to increased blood brain barrier (BBB) permeability, increased hippocampal neurogenesis, increased microglia activation, and the release of BDNF and chemokines (Silver and Curley et al., 2013). TNF-α, which is the only cytokine that MCs can store for rapid release and form through *de novo* synthesis, (Gu et. al. 2015) may increase and perpetuate neuroinflammation in the neonate brain (Dong et. al. 2014). All told, MC degranulation is capable of inducing a robust acute inflammatory response (Cocchiara et al., 1997).

While the interaction between ethanol and MCs is still unknown, we predict that ethanol activates neuroimmune cells through interactions with TLR-4 (Figure 1), which is found on the surface of MCs and microglia (as reviewed in Skaper et al., 2012, Crews et al.,

2012). This activation may occur through ethanol's direct binding of TLR-4, or through the upregulation of HMGB1, which agonizes TLR-4 and leads to enhancement of transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and stimulates the production and release of pro-inflammatory cytokines (Crews et al., 2015). Research has shown that TLR-4 signaling is implicated in MC activation (Xue et al., 2016). To our knowledge, no prior research has been conducted to determine whether third trimester-equivalent ethanol exposure leads to increased levels of MC degranulation or the mechanism by which this may occur.

In the current study, there were no significant treatment group differences in total MC numbers, either inside or outside of the dorsal and ventral hippocampus (Figures 4A, B, C). UC subjects had similar numbers of MCs in relation to SI-Veh, 5E-Veh, and 5E-Crom rats suggesting that intragastric intubations, ICV injections, ethanol exposure, and cromolyn administration had no significant effects on MC numbers. This signifies that no experimental manipulation increased the infiltration of central or peripheral MCs into the hippocampus or surrounding tissue. Across all groups, two trends remained constant: there were vastly more MCs outside than inside of the hippocampus, and there were more MCs in the dorsal hippocampus than the ventral hippocampus, which could lead to important findings in the future due to their functional differences (Figures 4C, D, E). Furthermore, there were significant increases in the number (Figure 4F) and percentage of degranulated MCs in and around the developing hippocampus in 5E rats (Figure 5A, B, C, D). Thus, postnatal ethanol exposure effectively doubled ($\sim 6\%$ to $\sim 12\%$) the percentage of degranulated MCs in 5E-Veh rats. These effects were found throughout every area analyzed in this study, both inside and outside of the dorsal and ventral hippocampus and were

significantly attenuated by ICV injections in 5E-Crom rats to levels roughly comparable to those found in controls (Figures 5A, B, C, D). Moreover, it may be important to use another marker for MCs, such as PGD₂, to identify fully degranulated MCs (O'Sullivan 1999), which may not be visible using toluidine blue as it stains only the MC's granule contents. Our results signify that MCs are, in fact, activated by early-life ethanol exposure, an effect that was reduced to baseline levels by the pre-treatment of cromolyn.

Microglia, the brain's primary resident immune cell, arise from the yolk-sac and are of myeloid origin (as reviewed in Harry and Kraft 2012). Microglia, as originally described by Virchow, can be detected within the rodent brain beginning on gestational day (GD) 10, at which point they begin to proliferate into the CNS, including the hippocampus (Harry and Kraft 2012). Ultimately, microglia comprise approximately 10% of the brain's total cell numbers (Harry and Kraft 2008). Microglia are found primarily in a quiescent, thin ramified state and upon activation become ameboid-like, mobilize, and phagocytose neuronal insults in order to mitigate damage (Nelson and Lenz 2017). Microglia, similar to MCs, express TLR-4 on their surface (Crews et al., 2012) and are activated by ethanol through a similar mechanism (detailed above). Microglia activation can result in the propagation or attenuation of neuroinflammation through the production of pro- and anti-inflammatory cytokines, ROS, and growth factors (Harry and Kraft 2008). In the past, research has shown that PD 4-9 ethanol exposure leads to increased neuroinflammation through the activation of microglia and the subsequent upregulation of proinflammatory cytokines, including IL-1 β and TNF- α (Drew et al., 2015).

While research describing the interactions between MCs and microglia is relatively new, many potential mechanisms exist for their bi-directional communication. Importantly,

the activation of TLRs on MCs results in cytokine release and the recruitment of microglia, which is also dependent on TLR signaling (as reviewed in Skaper et al., 2014). MC tryptase activates protease-activated receptor 2 (PAR-2) on microglia, leading to an increase in pro-inflammatory cytokine release and ROS (Zhang et al. 2012). MC release of different pro-inflammatory cytokines, chemokines, serotonin, and histamine can lead to the activation of microglia as well, (Silver and Curley 2013) and microglia may, in turn, stimulate MCs through the release IL-6 and TNF- α , which induces MC degranulation and the release of TNF- α (as reviewed in Skaper and Facci 2015). While many of these interactions are shown *in vitro* (Zhang et al., 2012; Zhang et al., 2016), few studies have shown potential interactions between MCs and microglia *in vivo* (Dong et al., 2016; Valle-Dorado et al., 2015).

In this study, postnatal ethanol exposure did not result in significant changes in overall hippocampal microglia numbers (Figure 6A), despite reports that show an increased neuroinflammatory response following postnatal ethanol exposure (Drew et al., 2015; Boschen et al., 2016). Such results suggest PD 4-6 ethanol does not lead to an overall increase in hippocampal microglia number, though it does induce microglia to transition to an amoeboid phenotype (Figure 6B). The significant attenuation of this effect by pre-treatment with cromolyn in 5E-Crom rats indicates that increases in microglia activation may result, in part, from the degranulation of MCs. Upon further investigation, and the analysis of three hippocampal sub-regions (CA1, CA3, and DG) the increase in amoeboid-like microglia was most prominently found in the DG of the dorsal and ventral hippocampus (Figures 7D, 8D), located adjacent to the velum interpositum, where the highest amount of MCs were amassed. In 5E-Veh rats increases in amoeboid-like microglia were found in area

CA1 of the dorsal hippocampus (Figure 7D), which were not attenuated by cromolyn administration in 5E-Crom rats. These findings suggest postnatal ethanol exposure may directly activate some proportion of microglia within the dorsal CA1, as the phenotypical activation of microglia is not reduced by cromolyn administration. These results support our hypothesis that PD 4-6 ethanol exposure leads to increased microglia activation, which is largely dependent on an upregulation in MC degranulation.

It is important to note that our experimental manipulations seem to have influenced the baseline activation state of microglia. Specifically, intubations and/or ICV injections in SI-Veh, 5E-Veh and 5E-Crom rats significantly altered the baseline activation states of microglia in comparison to UC subjects. There was a significantly larger percentage of thin microglia and a significantly smaller percentage of thick microglia (dorsal only) seen in all sub-regions of the developing hippocampus of UC rats when compared to SI-Veh, 5E-Veh, and 5E-Crom rats (Figures 6D, 7A, 7B, and 8A). The invasive nature of these procedures may have led to high stress levels and/or the initiation of neuroinflammation, as indicated by a non-significant increase of ameboid-like microglia in SI-Veh subjects when compared to controls which has been shown in previous studies (Boschen et al., 2016). To clarify these effects, it will be necessary to utilize SI rats that are not submitted to ICV injections, which will determine if the increase in inflammation is a result of the intubation or ICV injection procedures. In addition to an SI alone control group, a SI-Crom group could be used to determine if too much suppression of the neuroimmune system, through the stabilization of MCs, leads to negative effects. The impact of neuroimmune activation is similar to that of an inverted “U” scale, too much or too little MC activation can be detrimental (Silver and Curley 2013).

Results of this study suggest PD 4-6 ethanol exposure results in the increased morphological activation of microglia, a process potentially driven by the rapid activation of MCs. However, more research needs to be conducted to elucidate these findings. The current study used only male subjects, and microglia number and activation state are regulated in a sex-dependent manner, which suggests that there could be potential sex effects in MC-microglia interactions. In relevance to this study, microglia are found in higher numbers with increased activation within the hippocampus of PD 4 male rats relative to females (Schwarz et al., 2012; as reviewed by Nelson and Lenz 2017). Due to the differences in immune function between males and females (Nelson and Lenz 2017), it will be necessary to utilize female subjects in order to determine any potential sex effects of postnatal ethanol's activation of the neuroimmune system.

In addition, the morphological activation of microglia does not necessarily lead to an increase in pro-inflammatory signaling and neuroinflammation (Marshall et al., 2013). To study these effects in the current model, it will be important to investigate the expression of both pro- and anti-inflammatory cytokines induced by postnatal ethanol exposure through quantitative polymerase chain reaction (qPCR) and ELISA, to quantify gene and protein expression, respectively. If an increase in neuroinflammation is found, following postnatal ethanol exposure, it may suggest rapid MC degranulation amplifies or drives the morphological activation of microglia. Moreover, the development of feed-forward loops between the two neuroimmune cell types may contribute to the chronic nature of ethanol-induced neuroinflammation following postnatal exposure.

In fact, neonatal neuroinflammation in response to ethanol exposure has been demonstrated to contribute to later life cognitive dysfunction in rodents (Tiwari and

Chopra 2011). Thus, future work will investigate if cromolyn administration (at the time of ethanol exposure) can mitigate neuroinflammation through the stabilization of MCs and a reduction in microglia activation. Specifically, we posit the mitigation of ethanol-induced neuroinflammation can improve (or rescue) previously documented hippocampus-dependent learning and memory deficits in 5E rats (DuPont et al., 2014; Goodfellow et al., 2014). In total, results of this thesis suggest studying microglia in isolation may miss important therapeutic targets that arise from MC-microglia interactions.

In conclusion, this thesis investigated the effects of PD 4-6 ethanol exposure on hippocampal MC degranulation and microglia activation. Our results signify that postnatal ethanol exposure significantly increased the percentage of both MC degranulation and the morphological activation of microglia in the hippocampus of 5E-Veh rats. These results were significantly attenuated following pre-treatment with cromolyn in 5E-Crom rats. Results from this study are anticipated to significantly inform our understanding of postnatal ethanol's acute and chronic effects on the developing hippocampus and cognitive ability.

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